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ORIGINAL ARTICLE

Breakpoint mapping of 13 large *parkin* deletions/duplications reveals an exon 4 deletion and an exon 7 duplication as founder mutations

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Abstract Early-onset Parkinson's disease (EOPD) has been associated with recessive mutations in *parkin* (PARK2). About half of the mutations found in *parkin* are genomic rearrangements, i.e., large deletions or duplications. Although many different rearrangements have been found in *parkin* before, the exact breakpoints involving these rearrangements are rarely mapped. In the present study, the exact breakpoints of 13 different *parkin* deletions/duplications, detected in 13 patients out of a total screened sample of 116 EOPD patients using Multiple Ligation Probe Amplification (MLPA) analysis, were mapped using real time quantitative polymerase chain reaction (PCR), long-range PCR and sequence analysis. Deletion/duplication-specific PCR tests were developed as a rapid and low cost tool to confirm

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MLPA results and to test family members or patients with similar *parkin* deletions/duplications. Besides several different deletions, an exon 3 deletion, an exon 4 deletion and an exon 7 duplication were found in multiple families. Haplotype analysis in four families showed that a common haplotype of 1.2 Mb could be distinguished for the exon 7 duplication and a common haplotype of 6.3 Mb for the deletion of exon 4. These findings suggest common founder effects for distinct large rearrangements in *parkin*.

Keywords Parkinson's disease · *parkin* · Deletion · Duplication · Common founder · Breakpoint mapping

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder classically characterized by the clinical triad of bradykinesia, muscular rigidity and a resting tremor [1]. The mean age of onset for PD and early onset PD (EOPD) is 60 and 45 years, respectively [1, 2]. Autosomal recessive levodopa-responsive EOPD can be caused by mutations in *parkin (PARK2)*, *PINK1* or *DJ-1* [2, 3]. Mutations in *parkin* are the most common among the recessive forms of PD [2]. This gene encodes Parkin, an E3 ubiquitin ligase. As part of the ubiquitin proteosome pathway, Parkin can protect neurons from cellular insults like alpha-synuclein toxicity and protosomal dysfunction [4]. Recent findings revealed that Parkin might also be involved in the development and/or progression of ovarian and other cancers, suggesting that *parkin* might also be a tumor suppressor gene [5, 6].

Since the cloning of *parkin* in 1998, a wide variety of mutations has been identified in this gene [7, 8]. About 50% of the mutations found in *parkin* are small (point) mutations detectable by sequencing. The remaining 50% consists of genomic rearrangements, i.e., large deletions or duplications [9, 10]. This high rearrangement rate can be explained by the fact that *parkin* is located within the large common fragile site (CFS) *FRA6E* [11]. CFS's are reported to be highly susceptible to genomic instability [11]. Although little is known about the role of CFSs, many of the genes within these regions appear to function within the stress response system and in normal neurological development. The observation that extremely large genes, located in an unstable region, are highly evolutionary conserved suggests that these unstable regions and the genes within share a role in normal cell function [11].

Since genomic sequencing is not suitable to detect large rearrangements, other more quantitative methods are needed for a sensitive screening of these genomic variants. Available techniques include polymerase chain reaction (PCR)-based amplifications to detect homozygous deletions [3, 12], fluorescent dosage PCR [7], quantitative PCR (q-PCR) [8, 13–15], microarray [16] and Multiple Ligation Probe Amplification (MLPA) [17, 18]. MLPA is a rapid and sensitive technique to detect alterations in copy numbers using a multiplex PCR reaction. This method is suitable to identify deletions/duplications in *parkin* [17, 18]. Although many rearrangements have been found in *parkin*, the exact breakpoints involving these rearrangements are rarely mapped [3, 10, 12, 16]. In the present study, the breakpoint mapping of 13 different *parkin* deletions/duplications as found in 116 EOPD patients, is described. This approach enabled the development of specific deletion/duplication PCR's as a rapid and low cost tool to confirm MLPA results and to test family members or patients with similar *parkin* deletions/duplications.

Materials and methods

Study population

The study population consisted of 116 patients whose samples were sent to the DNA diagnostic laboratory (Department of Clinical Genetics, Erasmus Medical Centre, Rotterdam, The Netherlands) between March 12, 1999 and February 3, 2010 for genetic screening of *parkin*. Samples were sent by neurologists (n=89, 76.7%), clinical geneticists (n=17, 14.6%) or other medical specialists (n=10, 8.6%) because of a clinical diagnosis or symptoms of EOPD. The mean age of patients was 46.1 years (range 10–80 years) at time of blood sampling. The majority of the patients was male (n=71, 61%).

Mutation analysis

DNA was extracted from peripheral blood using the DNA Blood Kit Special (Chemagen, Baesweiler, Germany), which is based on DNA extraction and purification with magnetic beads [19]. All 12 coding exons and exon/intron boundaries of *parkin* (NM_004562.2, NCBI build 36 (UCSC hg18, March 2006)) were screened using direct sequence analysis. SALSA MLPA kits P051 and P052 (MRC Holland, Amsterdam, The Netherlands) were used to detect large rearrangements in *parkin*. MLPA analysis was performed according to manufacturer's instructions. MLPA products were run on an ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA, USA) and data was analyzed using Genemarker software version 1.5 (Softgenetics, State College, PA, USA).

q-PCR analysis

Real time quantitative PCR (q-PCR) was performed using Fam labelled Taqman assays, designed with Primer Express

version 2.0.0 (Applied Biosystems), around the deleted/duplicated exons of *parkin* (NM_004562.2, NCBI build 36 (UCSC hg18, March 2006)). All primers were tested for specificity by performing a BLASTn run. Taqman probes were designed with an 8–10°C higher melting temperature (T_m) than the primers. This was achieved by incorporating locked nucleic acid (LNA) monomers in the probe [20]. LNA containing probes have an enhanced hybridisation performance compared to classical Taqman probes and allow a shorter probe design [20]. T_m calculations for LNA incorporated probes were performed using the Exiqon T_m prediction tool (http://www.exiqon.com/ls/homeoflna/Oligo-tools/tm-prediction-tool.htm). LNA based Taqman assays were manufactured by Eurogentec (Seraing, Belgium). Gene dosage alterations were detected by performing a relative quantification run on an ABI7500 real-time PCR system (Applied Biosystems). Real-time PCR reactions were performed in a total volume of 25 µl, containing 20 ng DNA, 1× q-PCR mastermix Pluslow ROX (Eurogentec), 1× RNAse P (endogenous control) (Applied Biosystems), 30 µM forward and reverse primer and 10 µM probe. PCR conditions were initial 2 min 50°C incubation, followed by 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. All samples were analyzed in triplicate and compared to a normal control sample as calibrator [21].

Long-range PCR

Long-range PCR was performed using the Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN, USA). PCR products were sequenced using the ABI 3730XL automated sequencer (Applied Biosystems). Data was analyzed using the Sequencing Analysis software version 5.3.1 (Applied Biosystems).

Deletion/duplication specific PCR

For all deletions/duplications, a specific deletion/duplication-PCR test was designed using two primers (primers A and B) flanking the deletion/duplication and one internal primer (primer C; also see Table 1). Obtained PCR products run on a 1.5% agarose gel will show a mutated allele and a normal allele for a patient with a heterozygous deletion/duplication, a mutated allele only for a patient with a homozygous deletion/duplication and a normal allele only for a non-affected individual.

Haplotype analysis

Haplotypes were constructed using nine polymorphic microsatellite markers around the *parkin* locus. Three markers were located within the intronic regions of *parkin,* the remaining six markers were located 5′ and 3′ of *parkin* on chromosome 6 (see Fig. 1). Markers were amplified using standard conditions and reagents. Fam labelled PCR products were run together with a LIZ-500 size standard on an ABI 3730XL automated sequencer (Applied Biosystems). Marker allele sizes were determined using Genemarker software version 1.5 (Softgenetics).

Results

A total of 116 patients with clinical symptoms of EOPD were screened for mutations in *parkin* using direct sequencing and MLPA analysis. In 13 patients (11%), at least one pathogenic mutation was found (24 *parkin* mutations were found in total). Eighty three percent (n=20) of the pathogenic mutations consisted of large deletions/duplications, the remaining 17% (n=4) consisted of point mutations or small

Table 1 Primers used for deletion/duplication specific PCRs

Mutation	Primer A	Primer B	Primer C
Ex1del	tctttgactgcctttgcatga	tcacaactccctcaatgtcg	ccctttaaaacggtgctcag
Ex2dup	cccagttaccctgctgagaa	agaagctgggttaaggagcc	cttgctcttcacttcccagg
Ex2delA	ttttctgttgcatttggctg	gccttaccaccatttgctgt	gtgtcttcctcctggtttcg
Ex2delB	agccactggcatgttatcaa	tgagtggatgcattcagttctc	attggccatgttagggctc
Ex2delC	tccagcttttagccactcttc	caaccaaatctctgtatacccatca	aaatgactgatttcagaaggaaatg
Ex3delA	ccacctcttcatgcaatcac	ggcaacaactgtcaaacagc	aaattcttcagtgtagacaaacc
Ex3delB	ccagtgtggtgtgtgagtcc	tccaaagtggttccacatga	tgacaccaccatgctcactt
Ex4del	tacaagcttttaaagagtttcttgt	cgtggttcaatccctcttgt	aaaaccaactttgcgtcagg
Ex3-5del	cattgttttgaggggaaat	tcctcctaataatgataacaaagac	catatcatcaatgcgcacagaa
Ex5del	tctgaggctaggtcccttca	tgtctttcggcctcagtgtatcta	tcatgctgcattctggagac
Ex5-6del	ggaggggaggtgactttctt	gaggcgggtgagagattcta	catgtgtgggcatacctcat
Ex7dup	cagttcaaatccagtgttaccaa	aagcaatgggtcattccgta	ttccctttggtgcagtaacc
Ex8-10del	tgtcacccaagtgccagttact	tcagttgcttgtgcaacctc	ttcacctcacctctcccaac

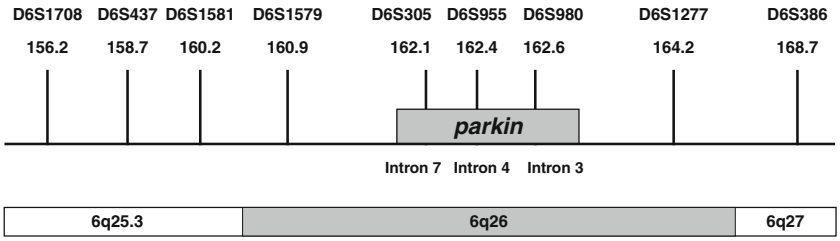

Fig. 1 Schematic overview (not drawn to scale) of the polymorphic microsatellite markers used for haplotyping. On *top of the bars* the polymorphic microsatellite markers are depicted with their chromosomal marker positions in Mb according to NCBI build 36 (UCSC hg18, March 2006)

deletions (e.g., a 2-bp deletion; data not shown). In Table 2, the characteristics of 12 families of the (index) patients with a large *parkin* deletion/duplication are summarized (families Tor-18-A and PV-24-A were reported before [22]). The remaining patient has one point mutation and was excluded from further analysis. Most of the patients with large deletions/duplications have classical parkinsonism syndromes like bradykinesia, hypokenetic rigidity and a resting tremor. The mean age of onset of the patients listed in Table 2 is 35.7 years. Table 3 gives an overview of all exon deletions and duplications found in our study population using MLPA analysis and breakpoint mapping.

To confirm the MLPA results with a different non-quantitative technique and to study recurrent deletions/duplications, the exact breakpoints of the rearrangements were mapped using q-PCR analysis; long-range PCR analysis and sequence analysis (see "Materials and methods" section).

Two examples of mapped rearrangements are shown in Fig. 2a (exon 4 deletion, Ex4del) and Fig. 2b (exon 7 duplication, Ex7dup). Ex4del also includes an insertion of 28 bp, whereas Ex7dup includes an insertion of 7 bp between the duplicated regions. The exact nomenclature of all deletions/duplications in this study is given in Table 3.

For each exon deletion/duplication, a specific deletion/duplication PCR test was developed based on the breakpoint analysis results. Results of the specific deletion/duplication PCR tests are shown in Fig. 3. Most patients show a heterozygous pattern (both deletion/duplication

Table 2 Characteristics of 12 families with a large *parkin* deletions/duplication

Family	Age of onset	Age at blood sampling	Mutation 1	Mutation 2	Occurrence	Clinical symptoms
A	48	74	Ex1del	Ex3-5del	S	Hypokinetic rigid syndrome, resting tremor, dyskinesia
B	58	59	Ex2dup[a]	R275W[a]	F	Rigidity, hypokinesia, resting tremor
C	28	30	Ex2Adel	Ex3Adel	F	Hypokinetic rigid syndrome, resting tremor, dopamine-sensitive
D	37	42	Ex2Bdel	Ex7dup	S	Bradykinesia in hand and legs, resting tremor
E	46	56	Ex2Cdel	N/P	S	Hypokinetic rigid syndrome, tremor, bradykinesia
F	38	42	Ex3Bdel	101delAG	F	Mild bradykinesia and rigidity, stooped posture, no dystonia
G	16	47	Ex3Bdel	R275W	S	Hypokinetic rigid syndrome, resting tremor arms and legs, dystonia
H II.1	45	45	Ex4del	Ex7dup	F	II.1 Hypokinetic rigidity, dystonia
II.3	38	42	Ex4del	Ex7dup		II.3 Parkinsonism, dystonia
II.5	33	39	Ex4del	Ex7dup		II.5 Parkinsonism, dystonia
I	20	50	Ex4del	Ex7dup	F	Hypokinetic rigid syndrome, dopamine-sensitive
J II.4	52	54	Ex5-6del	N/P	F	II.4: Tremor and rigidity right arm
III.1	22	47	Ex5-6del	Ex7dup		III.1: Tremor feet and right hand/rigidity
III.2	33	37	Ex5-6del	Ex7dup		III.2 Rigidity/dyskinesia
Tor-18-A	38	52	Ex5del	Ex5del	F	Bradykinesia, rigidity and tremor
PV-24-A	20	39	Ex8-10del	Ex8-10del	S	Bradykinesia, rigidity and tremor

S sporadic, *F* familial, *N/P* not present

[a] Located on the same allele

Table 3 Overview of the large *parkin* deletions/duplications found in this study

Family	Mutation	Exon	Del/dup	HGV nomenclature[a]
A	Ex1del	1	Del	c.-17782_7+123791del141580
B	Ex2dup	2	Dup	c.8-51502_172-33560dup198650insT
C	Ex2delA	2	Del	c.8-61987_171+4006del66157
D	Ex2delB	2	Del	c.8-48180_171+84346del132690
E	Ex2delC	2	Del	c.8-18045_172-62803del135951ins33
C	Ex3delA	3	Del	c.172-5243_412+18307del23791
F/G	Ex3delB	3	Del	c.172-4427_413-6108del59833
H/I	Ex4del[b]	4	Del	c.413-26603_534+367del27092ins28
A	Ex3-5del	3–5	Del	c.171+11347_618+17328del 395200
Tor-18-A[c]	Ex5del	5	Del	c.535-49422_619-12906del117274
J	Ex5-6del	5–6	Del	c.534+17825_735-64158del333241
D/H/I/J	Ex7dup	7	Dup	c.734+61166_871+2877dup129242ins7
PV-24-A[c]	Ex8-10del	8–10	Del	c.872-60873_1167+2393del245828

Del deletion, *dup* duplication

[a]Numbering according to NM_004562.2, NCBI build 36 (UCSC hg18, March 2006)

[b]Previously reported deletion [10]

[c]Previously reported families [22]

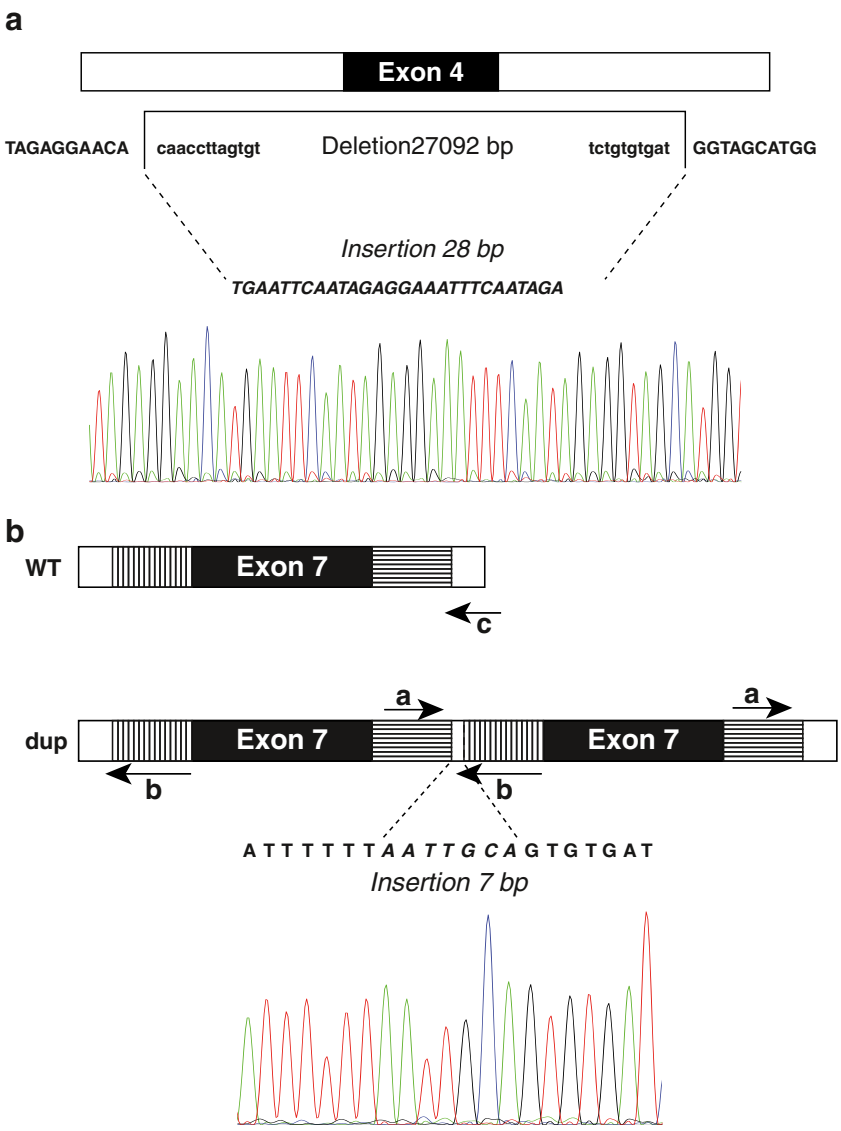

Fig. 2 Schematic overview of the breakpoint analysis results of Ex4del (A) and Ex7dup (B). Retained sequences are shown in *capitals*, deleted nucleotides are shown in *lower case*, inserted nucleotides are in *italic*. Indicated for Ex7dup are the 5′ (*vertically hatched*) and 3′ flanking regions (*horizontally hatched*) that are duplicated together with exon 7, and the primers (**a**, **b** and **c**) used for the specific duplication PCR test (see "Materials and methods"). *WT* wild-type, *dup* duplication

band and wild-type band present), whereas for Ex5del and Ex8-10del a homozygous pattern was observed (only mutant band visible).

Most of the exon deletions/duplications appear to be unique, but three exon deletions/duplications (Ex3delB, Ex4del and Ex7dup) were found among multiple families (see Table 3). Three index patients had an exon 2 deletion (Table 3; families C, D and E), but the breakpoints were different. Three index patients had an exon 3 deletion of which two types were found (Table 3; families C, F and G). Four index patients had the same Ex7dup (Table 3; families D, H, I and J), whereas 2/4 index patients also shared the same Ex4del (Table 3; families H and I). All available family members of families D, H, I and J were screened with the specifically developed deletion/duplication PCR test. These screening results are shown in Fig. 4.

Haplotypes were constructed around the *parkin* locus to investigate whether a common haplotype was present among the four families with the same Ex7dup mutation (Table 3; families D, H, I and J). Results of the haplotype analysis are presented in Fig. 4. For Ex7dup a shared haplotype was identified of at least 1.2 Mb as characterized by markers D6S1579 and D6S305. Ex4del shares a common haplotype of at least 6.3 Mb characterized by D6S955 to D6S386. Ex5-6del shares a common haplotype of at least 5.9 Mb characterized by D6S1708 to D6S305 found in one family only. Ex3del was found in two families (Table 3; families F and G), but for this mutation no common haplotype was found (data not shown).

Discussion

Over the past 11 years, 116 patients with early-onset Parkinson disease (EOPD) have been referred to our laboratory for genetic screening of *parkin*. Mutations were found in 13 patients with an overall mutation rate of 11%, which is slightly higher than reported before [14, 18, 23].

Multiplex Ligation-dependent Probe Amplification (MLPA) analysis was used in this study to screen for large deletions/duplications in *parkin*, because of its effectiveness to detect homo- and heterozygous deletions/duplications in *parkin* in a multiplex way. The MLPA technique is however prone to false positive results because of the possibility of polymorphisms on probe locations. Therefore, all deletions/duplications found need to be confirmed using a different technique [17, 23]. Our result that 83% of the mutations found in *parkin* consist of large deletions/duplications is higher than described previously [2, 14, 17, 18]. This finding clarifies that gene dosage studies are essential in *parkin* mutation screening. The high rate of large rearrangements might be explained by recurrent deletions/duplications in our study group or by the effectiveness of the MLPA technique to detect exon deletions/duplications compared to other techniques. Mapping of the exact breakpoints enables us to confirm MLPA results with a non-quantitative method and to compare the found deletions/duplications with other deletions/duplications in *parkin*. For all 13 different large deletions/duplications, a specific deletion/duplication-PCR test was developed as a low cost, quick, and robust technique to confirm MLPA results and to test other family members for the specific mutation or patients with similar MLPA results.

Only a few researchers have taken the effort to locate the exact breakpoints of large rearrangements found in *parkin* [3, 10, 12, 16], most likely because it is time-consuming as *parkin* is among the largest known genes with intronic regions up to 284 kb [11]. Four different exon 4 deletions were mapped before by two research groups [3, 10]. Ex4del in two of our patients (Fig. 2a) turned out to be the same deletion as reported by Hedrich et al. [10] in patients from Italy and Northern Germany. The compound heterozygous

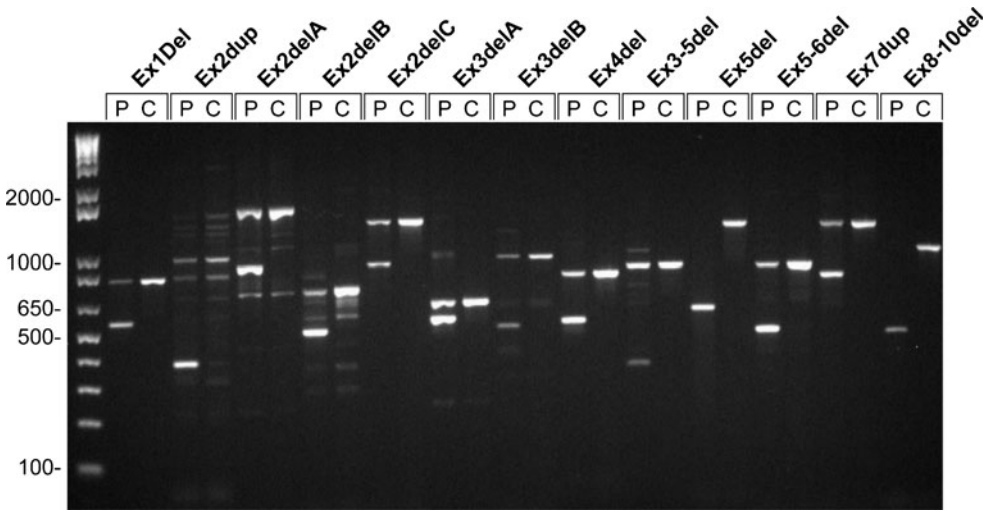

Fig. 3 Results of all 13 deletion/duplication specific PCR tests for patients (*P*) versus CEPH controls (*C*). Samples were run on a 1.5% agarose gel together with a 1-kb DNA ladder (first lane; Invitrogen, Carlsbad, CA, USA). PCR products of the mutated alleles are smaller than PCR products of the normal alleles. Some background PCR bands are visible for Ex2dup (above normal band of 791 bp) and Ex2delA (below mutant band of 873 bp)

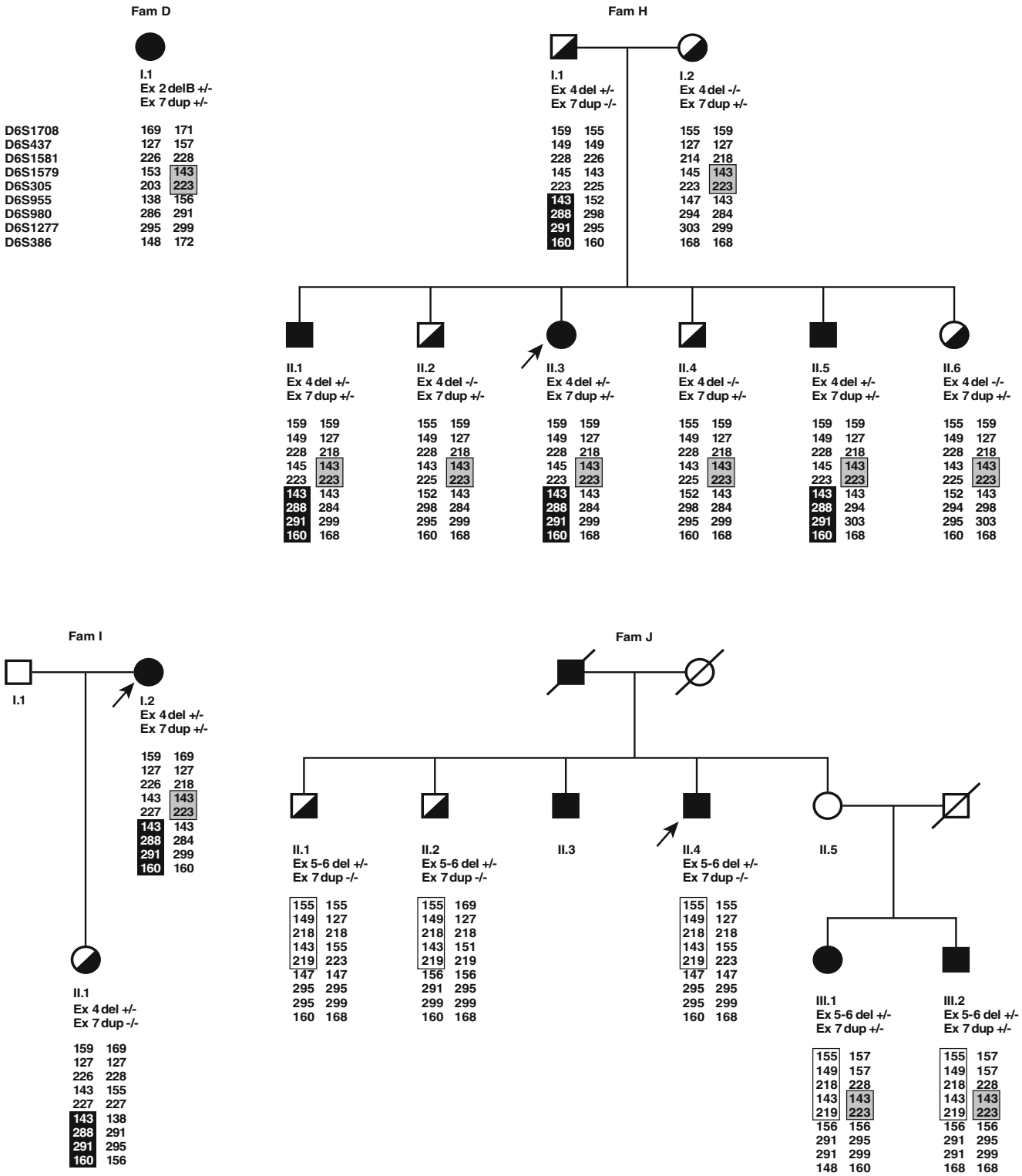

Fig. 4 Four EOPD pedigrees with the same Ex7dup. Individuals with EOPD are depicted with *filled symbols*, *half-filled symbols* represent carriers of one mutated allele and *open symbols* represent individuals with an unknown PD history. Index patients are indicated with an arrow. Results of the deletion/duplication specific tests are given for each individual as −/−; no deletion/duplication detected or +/−; heterozygous deletion/duplication. Also indicated for each individual are the nine markers with corresponding allele sizes. Common haplotypes for Ex4del, Ex5-6del and Ex7dup are depicted as a *black box*, *white box* or *grey box*, respectively

mutations of a deletion of exon 4 and a duplication of exon 7, as we found in families H and I, were reported previously in a Dutch patient [18]. In this patient, the reported mutations also occurred familial. Bayrakli et al. [16] mapped the breakpoints of an exon 5 deletion, but this deletion differs from the Ex5del found in our study. A duplication of exon 7 was reported by other researchers [7, 10, 18, 24–26], but was never mapped. Therefore, it remains undetermined whether these exon 7 duplications are identical. Most of the deletions/duplications listed in Table 3 are frameshift mutations that lead to a premature stopcodon several positions downstream. The only exception is Ex3-5del, which results in the loss of 149 residues. These residues are part of the Ubiquitin Like Domain (UBL) and the Src Homology 2 like (SH2-like) domain [6]. Although the exact function of these domains is still unclear, it is to be expected that the loss of 149 residues will lead to a loss of function of Parkin [6].

Similar haplotypes based on markers in the *parkin* region would support the hypothesis of a common founder effect multiple generations ago. The haplotype analysis (see Fig. 4) indicated that for Ex7dup a common haplotype of 1.2 Mb was present in four families, whereas a common haplotype of 6.3 Mb was present for Ex4del in two families. These data support the hypothesis of a common founder effect for mutations in *parkin* as reported before [10]. In autosomal recessive disease, founder mutations are commonly observed since in general there is no selection against carriers of mutations. Periquet et al. [27] reported a founder effect for some *parkin* point mutations, exon rearrangements were reported as independent recurrent events. It is unclear whether any of the exon rearrangements described by Periquet et al. are identical to ours since the deletions/duplications breakpoints were not mapped in their study.

A comprehensive literature overview among 379 unrelated carriers of *parkin* mutations showed a relatively high rate of exon deletions and duplications between exons 2 and 9 of *parkin* [10]. This is in line with the finding that the most unstable region in the CFS *FRA6E* was determined between exons 2 and 8 of *parkin*, suggesting hotspots for forming gaps and breaks in the large intronic regions [5]. In contradiction to this finding we found that in some cases (families D/H/I/J for Ex7dup; families F/G for Exdel3B and families H/I for Ex4del) deletions/duplications shared similar breakpoints (see Table 3), indicating a common founder effect. Our data provides evidence that, besides mutational hotspots, also common founder mutations are a source of exon rearrangements in *parkin*.

The *parkin* mutations found in pedigrees D, H and I follow a recessive inheritance pattern. In family J the difference between the affected patient II.4 and the unaffected individuals II.1 and II.2 who all share the same ex5-6del and are about the same age, remains unclear. The affected patient II.4 was negative for mutations in *LRRK2, SNCA, PINK1* and *DJ-1*. It is possible that this patient has a second yet undiscovered mutation in a PD related gene. It is also possible that a single heterozygous mutation in a recessive gene like *parkin* is sufficient to cause PD or at least to increase the risk of developing the disease [2]. Individuals II.3 and II.5 of family J and individual I.1 of family I were unfortunately not available for study. For family D, only the index patient was available for testing.

In conclusion, with the development of multiplex screening techniques like MLPA, an increasing amount of rearrangements will be found in EOPD patients. In the present study we demonstrate that breakpoint mapping of *parkin* deletions/duplications can be useful to compare exon rearrangements between patients and families and to study a possible founder effect. Using this approach we have identified common haplotypes for an exon 7 duplication and an exon 4 deletion. Furthermore, we also show that deletion/duplication-specific PCR tests can be developed as a rapid and low cost tool to confirm MLPA results and to test family members or patients with similar *parkin* deletions/duplications.

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